Neuron, Vol. 12, 1353–1362, June, 1994, Copyright © 1994 by Cell Press

Embryonic Expression of Myelin Genes: Evidence for a Focal Source of Oligodendrocyte Precursors in the Ventricular Zone of the Neurol Tube

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Summary

2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP) is an abundant protein of myelinating oligodendrocytes. We report that one of the alternatively spliced CNP mRNAs is also expressed in cultured oligodendrocyte progenitor cells. In situ hybridization revealed a thin longitudinal column of CNP-positive cells in the ventral ventricular zone of the embryonic day 14 rat spinal cord, coincident in time and space with cells that express the platelet-derived growth factor α receptor, another putative marker of the oligodendrocyte lineage. These data support the hypothesis that the oligodendrocyte lineage originates at a discrete location in the ventral ventricular zone of the embryonic day 14 rat spinal cord. We further report that transcripts encoding the myelin proteolipid protein (PLP/DM-20) are expressed in an unidentified population of neural progenitors in the ventricular zone abutting the floor plate. Our results support the idea that the ventricular zone is a mosaic of specialized progenitor cells.

Introduction

Myelin has a similar structure in the CNS and PNS and contains overlapping but nonidentical sets of structural proteins. The most abundant protein of CNS myelin is proteolipid protein (PLP), a transmembrane protein that accounts for about half of the total CNS myelin protein content. PLP is also expressed in the PNS, but is localized to Schwann cell bodies and is excluded from compact myelin (Kamholtz et al., 1992; Puckett et al., 1987). Myelin basic protein (MBP) is a major constituent of CNS myelin that is also expressed, at a lower level, in PNS myelin. In addition, there are polypeptides that, although they are enriched in myelin membrane preparations, are not necessarily structural components of the myelin sheath. One of these is the enzyme 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNP; Vogel and Thompson, 1988), which occurs in both the CNS and PNS.

It is now known that some of these myelin proteins are expressed, not only in myelinating oligodendrocytes and Schwann cells, but also in the premyelinating CNS and PNS and even outside of the nervous system. For example, an alternatively spliced isoform of PLP known as DM-20 (Nave et al., 1987) is expressed at low levels in the embryonic and perinatal rodent brain and spinal cord (LeVine et al., 1990; Ikenaka et al., 1993; Timsit et al., 1992) and in the developing mouse heart (Campagnoni et al., 1992). Alternatively spliced mRNAs encoding CNP (Bernier et al., 1987; Kurihara et al., 1990) have been detected in circulating lymphocytes, erythrocytes, and platelets (Bernier et al., 1987; Sprinkle et al., 1985), as well as in premyelinating nervous tissue (Timsit et al., 1992; Scherer et al., 1994 [this issue of *Neuron*]; this paper). Some MBP-related mRNAs and proteins are also expressed in the developing CNS before the onset of myelination (Sorg et al., 1987; Verity and Campagnoni, 1988). Thus, some of the myelin proteins may perform additional functions not directly related to myelination in both neural and nonneural cells.

We considered the possibility that the presence of myelin gene products in the embryonic CNS might be due to precocious low level expression of the myelin genes in oligodendrocyte progenitor cells. If so, one or more of the myelin gene products might serve as a marker for oligodendrocyte precursors in situ and might be useful for tracing the sites of origin and migration routes of these cells during development. We examined the expression of CNP, PLP/DM-20, and MBP mRNAs in cultured oligodendrocyte progenitor cells (O-2A progenitors) that were maintained in the presence of platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), which together keep these cells dividing and prevent oligodendrocyte differentiation in vitro (Bögler et al., 1990). We found that dividing O-2A progenitors express CNP mRNA, although they do not appear to express significant amounts of PLP/DM-20 or MBP mRNAs.

By in situ hybridization, we could detect CNP expression in the developing spinal cord as early as embryonic day 12 (E12), at which time CNP transcripts were uniformly expressed throughout most of the ventral half of the cord. By E14, however, the zone of CNP gene expression had regressed to a thin longitudinal column of cells in the ventricular zone in the ventral half of the spinal cord. These CNP-positive cells colocalize in time and space with cells that express mRNA encoding the PDGF α receptor (PDGF-αR), another putative marker of oligodendrocyte progenitors (Pringle et al., 1992), reinforcing our previous suggestion that the oligodendrocyte lineage originates in a specialized part of the ventricular zone in the ventral half of the spinal cord (Pringle and Richardson, 1993). Transcripts encoding PLP/DM-20 were also localized to a small section of the ventricular zone but earlier, and more ventral, than the CNP expression domain. Therefore, PLP/DM-20 and CNP appear to be expressed by different subsets of progenitor cells in the embryonic rat neural tube.

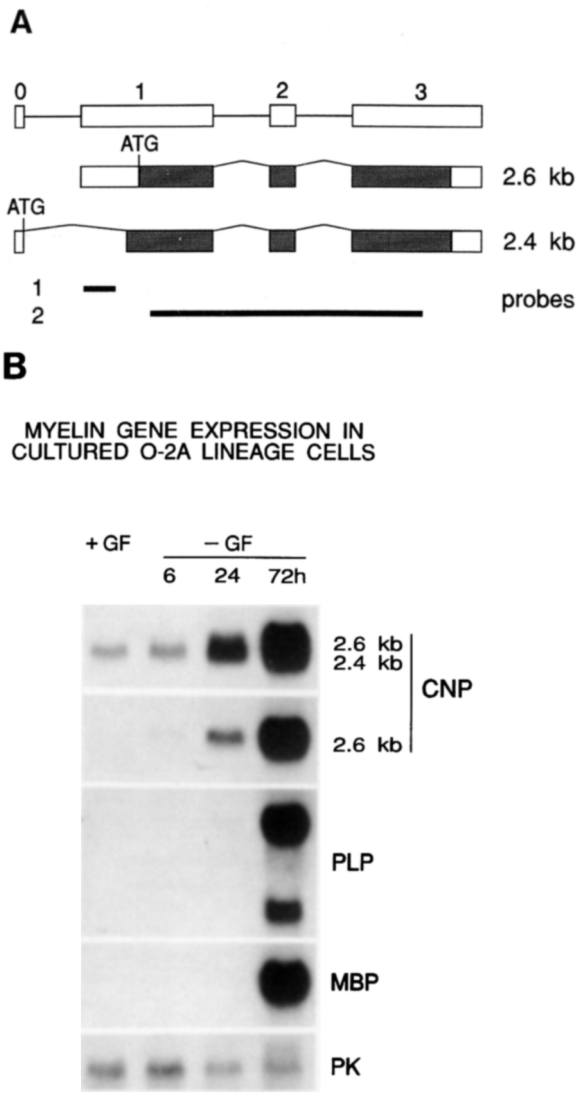

Figure 1. Expression of CNP Transcripts in Undifferentiated Oligodendrocyte Precursors

(A) Alternatively spliced transcripts encoding CNP and the probes used to detect them (adapted from Kurihara et al., 1990). The CNP gene structure is shown at the top (not to scale). Numbered boxes represent exons. Beneath this are diagrams of the two major CNP mRNAs, which are produced from primary transcripts with different 5′ ends (Bernier et al., 1987; Kurihara et al., 1990) and encode proteins with different amino termini. The larger CNP mRNA encodes the smaller protein (CNPI), and the smaller mRNA encodes the larger protein (CNPII) (Kurihara et al., 1992). Shaded boxes, coding sequence. Unshaded boxes, 5′ and 3′ noncoding sequences.

(B) Northern blot analysis of mRNA from cultures of immunoselected O-2A progenitors, grown in the presence of PDGF-AA and bFGF (+GF) or in medium lacking these growth factors (−GF) for the indicated periods of time. The blot was sequentially probed for CNP transcripts (probe 2; see Figure 1A), then specifically for the larger CNP transcript (probe 1; Figure 1A), MBP transcripts, pyruvate kinase (PK) transcripts (a control for gel loadings), and PLP/DM-20 transcripts. Proliferating O-2A progenitor cells (+GF) express the smaller, 2.4 kb CNP transcript, but little or no 2.6 kb CNP transcript, MBP transcript, or PLP/DM-20 transcripts. In each case, the autoradiographic exposure was between 3 and 4 days, except for MBP in which the exposure was 24 hr.

Results

Proliferating Oligodendrocyte Progenitor Cells Express CNP In Vitro

We purified O-2A progenitor cells from monolayer cultures of neonatal rat cerebral cortex by immunoselection (Collarini et al., 1992) with monoclonal antibody A2B5 and cultured them in defined medium supplemented with 0.5% fetal calf serum and 10 ng/ml each of PDGF and bFGF. This combination of growth factors keeps O-2A progenitor cells proliferating and prevents oligodendrocyte differentiation in vitro (Bögler et al., 1990). While the cells were still subconfluent, PDGF and bFGF were withdrawn from the culture medium to induce the O-2A progenitors to cease dividing and start to differentiate synchronously into oligodendrocytes (Collarini et al., 1992). Total cellular RNA was prepared from replicate cultures at various times after growth factor withdrawal and analyzed on Northern blots.

Using a probe generated from a full-length rat CNP cDNA (probe 2; Figure 1A), we detected two CNP mRNAs of approximately 2.4 kb and 2.6 kb in our cultured cells. CNP transcripts of these sizes have also been found in mRNA prepared from embryonic and postnatal rat brain tissue (Bernier et al., 1987; Kurihara et al., 1990; Scherer et al., 1994). The 2.4 kb transcript was present in O-2A progenitors that were maintained in a proliferating state by PDGF and bFGF (Figure 1B, +GF) and was up-regulated within 24 hr of growth factor withdrawal, at which time the 2.6 kb transcript was also apparent (Figure 1B, −GF). Between 24 and 72 hr after growth factor withdrawal, both transcripts were further up-regulated. The 2.4 kb and 2.6 kb CNP mRNAs are known to be derived from the same gene by alternative splicing (Bernier et al., 1987; Kurihara et al., 1990). A hybridization probe specific for the 2.6 kb mRNA (probe 1; Figure 1A) detected CNP mRNA only in differentiating oligodendrocytes, confirming that proliferating O-2A progenitor cells express predominantly the 2.4 kb CNP mRNA (Figure 1B). The same blot was reprobed for PLP/DM-20 and MBP transcripts. PLP and DM-20 transcripts differ by the presence (PLP) or absence (DM-20) of sequences derived from an alternative 105 bp exon (Nave et al., 1987). This size difference cannot be resolved on our Northern blots. The ~3.3 kb and ~2.3 kb RNA transcripts that we detect with our PLP/DM-20 probe are generated by alternative poly(A) site selection (Milner et al., 1985) and represent overlapping PLP- and DM-20-specific mRNAs. These transcripts were detected in differentiating O-2A cultures that had been maintained in the absence of growth factors for 24 hr and were strongly up-regulated between 24 and 72 hr after growth factor withdrawal (Figure 1B), but no PLP-related transcripts could be detected in O-2A progenitor cells that were proliferating in the presence of PDGF and bFGF (Figure 1B, +GF). Neither were MBP-related transcripts detected in cycling O-2A progenitors, although a sin-

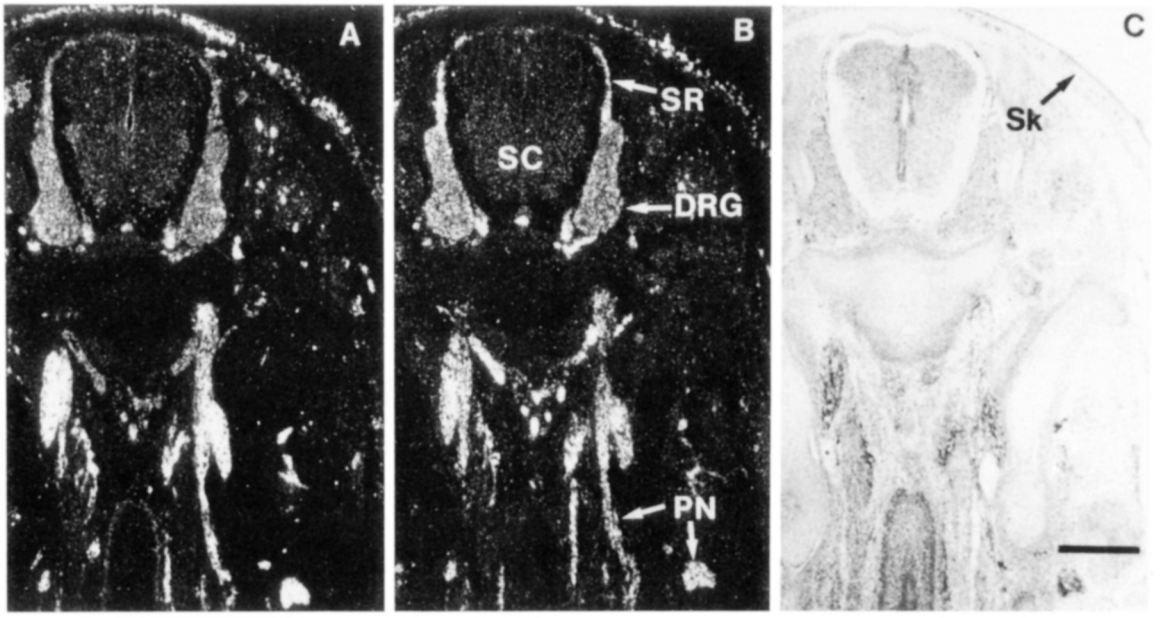

Figure 2. Visualization of CNP and PLP/DM-20 Transcripts in an E16 Rat Embryo by In Situ Hybridization

(A) CNP probe, dark-field illumination. (B) PLP/DM-20 probe, dark-field illumination. (C) Bright-field micrograph. Positive hybridization is evident throughout the PNS in both (A) and (B): dorsal root ganglia (DRG), spinal roots (SR), many peripheral nerves (PN) sectioned transversely and longitudinally, and the skin (Sk) of the embryo. SC, spinal cord. The CNP and PLP/DM-20 expression patterns in the PNS are very similar in these consecutive sections. Autoradiographic exposure, 5 weeks. Bar, 0.5 mm (C).

gle, abundant ~3 kb MBP transcript appeared between 24 and 72 hr after growth factor withdrawal (Figure 1B).

Spatially Restricted Expression of CNP mRNA in the Embryonic Rat Spinal Cord

Since CNP transcripts could be detected in oligodendrocyte progenitor cells in culture, we were interested to see whether CNP transcripts could also be detected in situ in the embryonic CNS before the appearance of myelinating oligodendrocytes. Previous in situ hybridization studies of CNP expression have concentrated on postnatal development, during the period of oligodendrocyte differentiation (Jordan et al., 1989; Trapp et al., 1988).

We chose to focus on spinal cord development since this is a topographically simple part of the CNS and also because we could directly compare the CNP expression pattern with that of the PDGF-αR, which our previous studies indicated might be another marker of oligodendrocyte precursors in the CNS (Pringle et al., 1992; Pringle and Richardson, 1993). We cut transverse sections through the lumbar, thoracic, and cervical regions of E12, E13, E14, E16, and E18 rat embryos as well as newborn, postnatal day 10 (P10), and P20 rat pups (gestation in the rat is 21 days so that the day of birth is designated E21/P0). The sections were subjected to in situ hybridization with a ^{35}S-labeled RNA probe transcribed in vitro from a full-length rat CNP cDNA. This probe does not discriminate between the 2.4 kb and 2.6 kb CNP transcripts. At all ages examined, CNP transcripts could be detected in the PNS, including the dorsal root ganglia, spinal nerve roots, and other peripheral nerves, and nerve target fields such as the skin (e.g., Figure 2). CNP transcripts were also detected in the CNS at all ages examined. In the E12 spinal cord, there was a low level of CNP expression in the ventral half of the cord, the dorsal boundary corresponding approximately to the sulcus limitans (Figure 3A). Within the ventral half of the cord at E12, CNP was expressed in both the ventricular zone (dividing cells) and the ventral horns (postmitotic motor neurons), but not in the floor plate or the ventricular zone immediately adjacent to the floor plate. Two days later, at E14, the CNP expression domain had regressed and was restricted to a ribbon of cells running along the length of the spinal cord in the ventral ventricular zone (Figures 3B and 3C). In cross-section, the longitudinal column of CNP-positive cells in the cervical region of the E14 cord appeared as two small foci of two or three cells, one each side of the central canal (Figure 3C). In the thoracic region of the E14 cord, where development is slightly delayed relative to more anterior regions, the column of CNP-positive cells had not yet regressed as fully as at the cervical level of the cord in the same animal (compare Figures 3B and 3C). Two days later, at E16 (data not shown), the foci of CNP expression had gone from the ventricular zone, and instead there was a speckled appearance to the

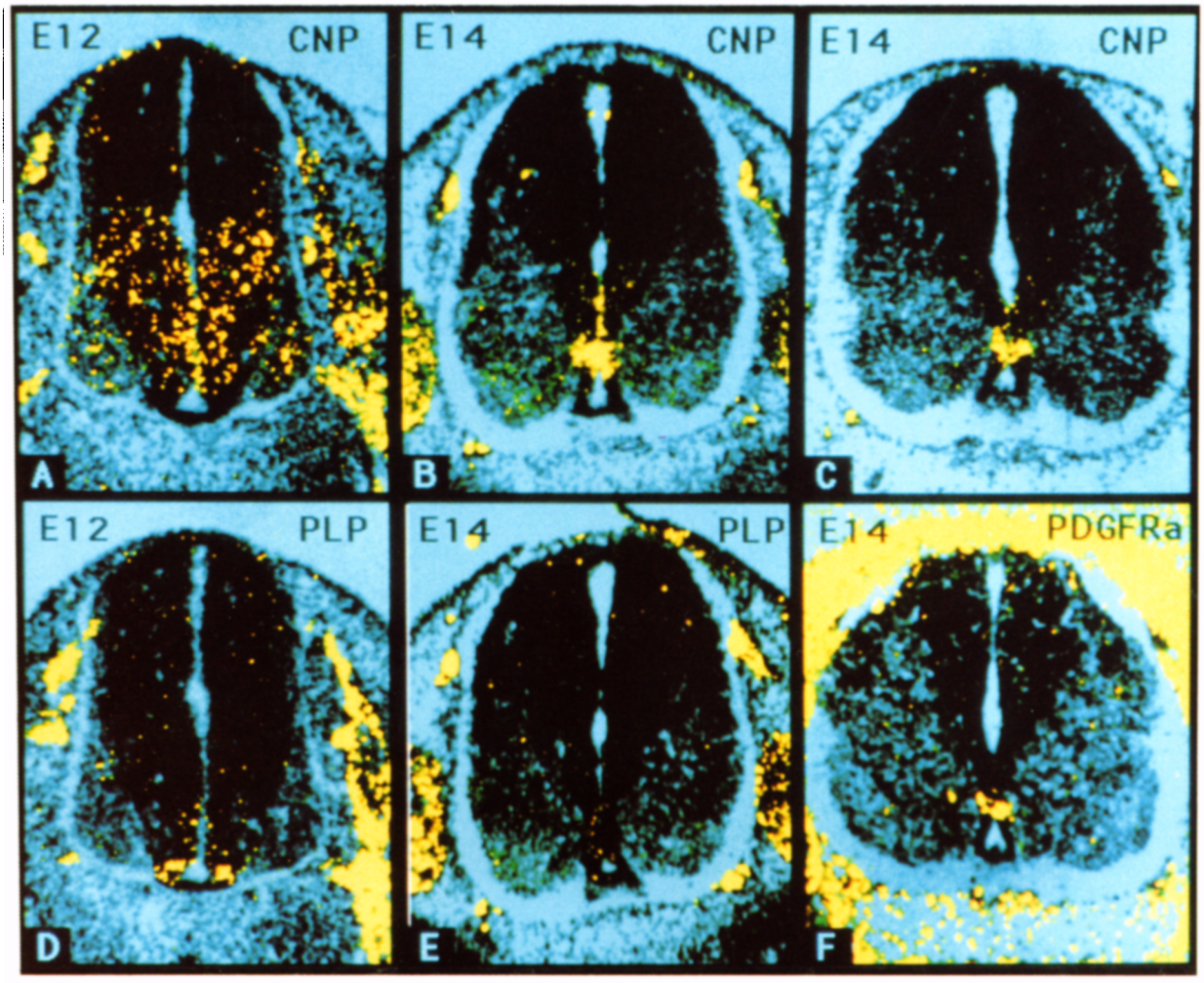

Figure 3. CNP, PLP/DM-20, and PDGF-αR Transcripts in Transverse Sections through the Spinal Cords of E12 and E14 Rat Embryos, Visualized by In Situ Hybridization

The ages of the animals and the probes used are indicated in the figure. (A) and (D) are consecutive sections from the thoracic region of an E12 embryo, (B) and (E) are consecutive sections from the thoracic region of an E14 embryo, and (C) and (F) are from the equivalent cervical region of age-matched E14 littermates. Images are computer-generated superimpositions of corresponding bright-field (blue) and dark-field (yellow) micrographs. Autoradiographic exposures: 5 weeks (A–E); 2 weeks (F).

surrounding area, suggesting that individual cells in the gray matter of the spinal cord were expressing low levels of CNP mRNA, close to the limit of sensitivity of our in situ hybridization procedure. After another 2 days, at E18 (Figure 4A), the same speckled pattern prevailed in the gray matter throughout the spinal cord, and, in addition, there were a few very intensely labeled cells in the ventral-most white matter and the adjoining gray matter (Figures 4A–4C, arrows). The contrast in signal intensity was marked between these latter, intensely labeled cells, which presumably represent the earliest differentiating oligodendrocytes in the spinal cord, and the weakly labeled cells present before E18; whereas the intensely labeled cells were clearly visible, even in bright-field illumination, after an autoradiographic exposure of 2 or 3 days, the weakly labeled cells required from 4 to 6 weeks and were only visible under dark-field illumination. In situ hybridization with a control probe that was homologous to the mRNA ("sense" probe) rather than complementary ("antisense" probe) gave no signal above background in this or any other of the experiments reported here (data not shown).

The very restricted pattern of CNP expression in the ventricular zone at E14 was similar to the pattern we had obtained previously using a probe for PDGF-αR, another putative marker for oligodendrocyte progenitors in the CNS (Pringle et al., 1992; Pringle and Richardson, 1993). We therefore compared the CNP expression pattern in the E14 cervical spinal cord with the PDGF-αR expression pattern in the equivalent region of an age-matched littermate. The positions of the foci of CNP and PDGF-αR expression were coincident, within the limits of our experiments (compare

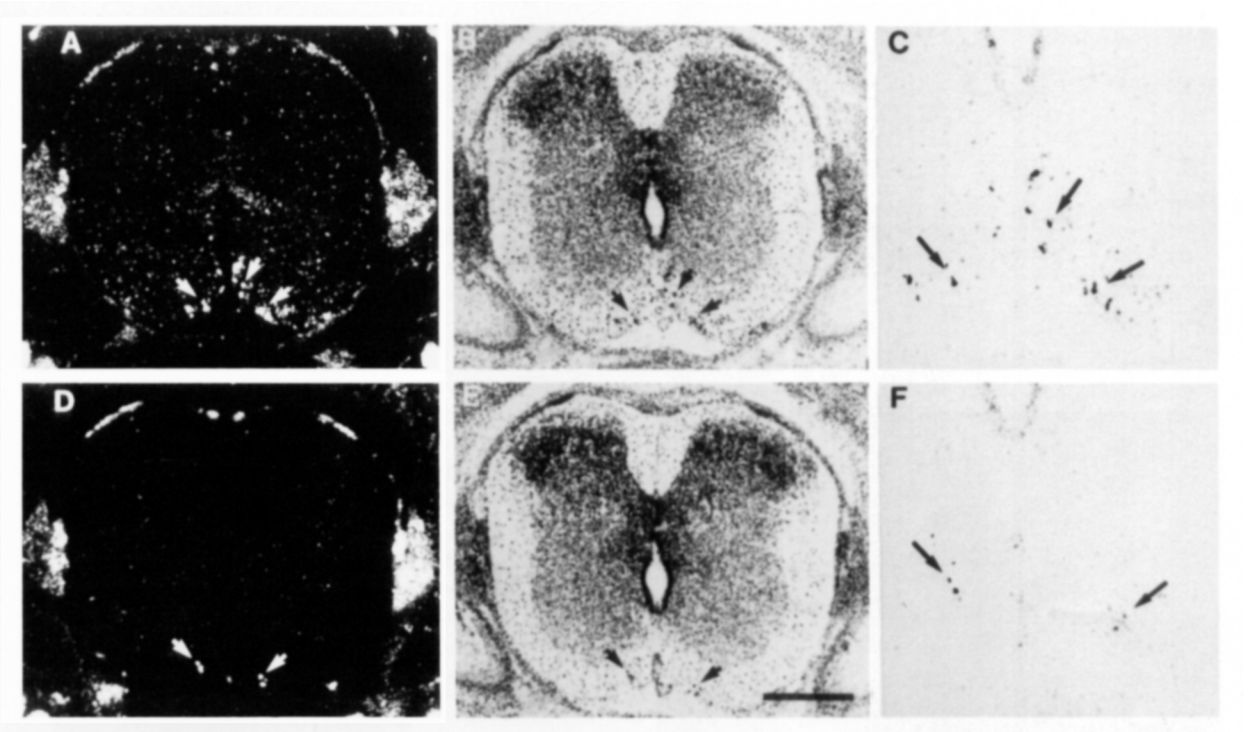

Figure 4. CNP and PLP/DM-20 Transcripts in Transverse Sections through an E18 Rat Spinal Cord, Visualized by In Situ Hybridization

(A–C) CNP probe. Many weakly CNP-positive cells can be seen throughout the gray and white matter of the E18 cord in dark-field illumination (A), whereas a few very intensely CNP-positive cells can be seen in the ventral-most white matter (arrows). These intensely CNP-positive cells can be seen after a few days' exposure even in bright-field illumination (B and C).

(D–F) PLP/DM-20 probe. In the E18 spinal cord, a very small number of strongly PLP/DM-20-positive cells can be seen (arrows) in either dark-field (D) or bright-field (E and F) illumination. These strongly PLP/DM-20-positive and the strongly CNP-positive cells presumably represent the first differentiating oligodendrocytes in the cord.

Sections presented in (A)–(C) and (D)–(F) are consecutive. (C) and (F) are higher magnification, higher contrast views of the ventral white matter illustrated in (B) and (E), respectively. Autoradiographic exposure, 5 weeks (but note that the intensely labeled cells were visible after only a few days).

Bar, 0.5 mm (E).

Figures 3C and 3F). This position was approximately 0.15 of the way along the dorsoventral axis, measuring along the lumen of the spinal cord in a ventral-to-dorsal direction.

In the postnatal brain and spinal cord, intensely CNP-positive cell bodies accumulated progressively, mainly in developing white matter and nearby gray matter, as described in previous studies (Jordan et al., 1989; Trapp et al., 1988). For example, the foliar white matter of the P10 cerebellum was filled with CNP-positive cells, as was the corpus callosum and various white matter tracts in the brain stem (Figures 5A and 5B). These CNP-positive cells presumably represent maturing oligodendrocytes.

Embryonic Expression Pattern of PLP/DM-20 Transcripts in the Spinal Cord

mRNA transcripts encoding DM-20, an alternatively spliced isoform of PLP that is missing 35 amino acids from one of the extracellular loops of PLP (Nave et al., 1987), have been reported to be expressed in the embryonic CNS and PNS (Ikenaka et al., 1993; Timsit et al., 1992) and outside the nervous system as well (Campagnoni et al., 1992). For example, one study has shown that DM-20 transcripts are expressed in the basal ventricular and subventricular zones of the developing mouse diencephalon at E10 (Timsit et al., 1992). We performed in situ hybridization with a PLP probe (one that does not distinguish PLP and DM-20 transcripts) on transverse sections of rat embryos at ages from E12 onwards. At all ages examined, we observed PLP/DM-20 expression in the PNS just as for CNP. We also observed bilateral expression of PLP/DM-20 along the length of the spinal cord at E12, restricted to a small region of the ventricular zone just above the floor plate (Figure 3D). These columns of PLP/DM-20-positive cells were clearly distinguishable from the columns of CNP-positive or PDGF-αR-positive cells described above (Figures 3C and 3F) because the PLP/DM-20-positive columns were present earlier (E12 rather than E14) and were located more ventrally. Between E12 and E16, we were unable to detect PLP/DM-20 expression in the spinal cord, even after long (5 week) exposure times (e.g., Figure 3E). At E18, a few intensely labeled cell bodies appeared in the most ventral white matter (Figures 4D–4F). These grew in

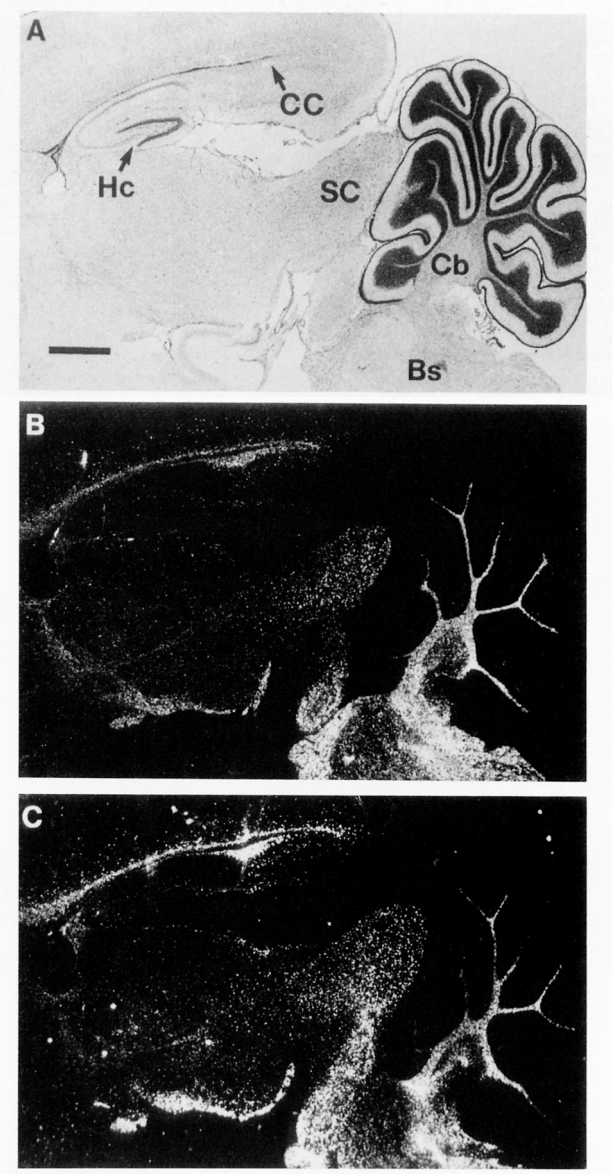

Figure 5. CNP and PLP/DM-20 Transcripts in Sagittal Sections through a P10 Brain and Cerebellum, Visualized by In Situ Hybridization

(A) Bright-field illumination.

(B) In situ autoradiograph with CNP probe, dark-field illumination. Intensely CNP-positive cells can be seen throughout the brain, especially in the corpus callosum (CC), superior colliculus (SC), brain stem (Bs), and in the foliar white matter of the cerebellum (Cb). Hc, hippocampus.

(C) PLP/DM-20 probe. Intensely PLP/DM-20-positive cells are found in the brain and cerebellar white matter in a similar distribution to the CNP-positive cells. Autoradiographic exposure, 1 week.

Bar, 1 mm (A).

number and spread through the white matter of the cord between E18 and P0 (data not shown). These strongly PLP/DM-20-positive cells, like the strongly CNP-positive cells, presumably represent the first differentiating oligodendrocytes in the spinal cord. Indeed, after E18, the expression patterns of PLP/DM-20 and CNP were virtually indistinguishable throughout the CNS (Figures 5B and 5C).

Discussion

Oligodendrocytes develop from glial progenitor cells known as O-2A progenitors (for reviews see Raff, 1989; Richardson et al., 1990; Pfeiffer et al., 1993). In vitro, O-2A progenitors are bipotential and can give rise to type-2 astrocytes or oligodendrocytes, depending on the composition of the culture medium. There is no firm evidence for the existence of type-2 astrocytes in vivo, however. O-2A progenitors are migratory cells in vitro, and there is evidence that they can migrate quite large distances in vivo during development or when transplanted into a myelin-deficient host (Warrington et al., 1993; Vijnais et al., 1993). In the developing rat optic nerve, for example, O-2A progenitors accumulate between E18 and P5 in a wave progressing from the optic chiasm toward the eye, suggesting that these cells are generated somewhere in the brain and subsequently migrate into the nerve (Small et al., 1987).

Oligodendrocyte Precursors First Arise in the Ventral Half of the Spinal Cord

There is evidence that oligodendrocytes in the rat spinal cord develop from precursor cells which originate in the ventral half of the cord and migrate dorsally. Warf et al. (1991) dissociated dorsal and ventral spinal cord cells from embryonic rats and cultured them separately in vitro. They found that oligodendrocytes developed on schedule in cultures of E14 ventral cells, but not in cultures of E14 dorsal cells. In contrast, oligodendrocytes developed in cultures of both E16 ventral and E16 dorsal cells. This is consistent with the idea that oligodendrocyte precursors originate in the ventral half of the E14 rat spinal cord and migrate into the dorsal half by E16. Further evidence for migration of oligodendrocyte precursors comes from the experiments of Noll and Miller (1993), who pulsed E16.5 rat embryos in utero with bromodeoxyuridine to label dividing cells. The majority of labeled spinal cord cells lay just outside the ventricular zone in the ventral half of the cord. Following a 2 day chase period, many bromodeoxyuridine-labeled cells were found in the lateral and dorsal regions of the cord, and the majority of the ventrally derived bromodeoxyuridine-labeled cells at E16.5 differentiated into oligodendrocytes when they were removed from the animal and cultured under conditions that promote oligodendrocyte differentiation in vitro (Noll and Miller, 1993).

A Focal Source of Oligodendrocyte Progenitors in the Ventral Ventricular Zone of the Embryonic Spinal Cord

Previous work from our laboratory has also suggested a ventral origin for oligodendrocyte precursors in the

embryonic rat spinal cord. By in situ hybridization, we visualized mRNA encoding PDGF-αR, a putative marker of the oligodendrocyte lineage, and discovered a narrow column of PDGF-αR$^+$ cells in the ventricular zone in the ventral half of the spinal cord of the E14 rat (Pringle and Richardson, 1993). In cross-section, this column initially contained just two cells on either side of the central canal. Subsequently, the PDGF-αR$^+$ cells increased in number and spread throughout the cross-section of the spinal cord, apparently having migrated away from their germinal site in the ventricular zone. Our evidence that these PDGF-αR$^+$ cells in the developing spinal cord represent oligodendrocyte precursors is as follows. First, O-2A progenitor cells from various regions of the developing CNS are known to express PDGF-αR in vivo (Hart et al., 1989) and in vitro (Hart et al., 1989; McKinnon et al., 1990) and proliferate in vitro in response to PDGF (McKinnon et al., 1990; Raff et al., 1988; Pringle et al., 1989; Richardson et al., 1988; Noble et al., 1988). Second, O-2A progenitors appear to be the only glial cells in cultures of perinatal rat optic nerve (Hart et al., 1989) or embryonic rat spinal cord (A. Hall and W. D. R., unpublished data) that express PDGF-αR. Third, the way that PDGF-αR$^+$ cells accumulate in the cerebral cortex, cerebellum, optic nerve, and spinal cord during development is consistent with what is known from other studies of oligodendrocyte development in these regions of the CNS (Pringle et al., 1992; Mudhar et al., 1993; Pringle and Richardson, 1993). In the developing optic nerve, for example, the first PDGF-αR$^+$ cells appear near the optic chiasm and subsequently accumulate throughout the nerve in a wave progressing toward the eye, reaching the lamina cribosa (a glial barrier that prevents oligodendrocytes or their progenitors from invading the retina; ffrench-Constant et al., 1988) around P5 (Mudhar et al., 1993); this mirrors in time and space the way that O-2A progenitors are thought to migrate into the optic nerve during development (Small et al., 1987). Fourth, the absolute number of PDGF-αR$^+$ cells in the perinatal rat optic nerve (Pringle et al., 1992) matches independent estimates of the number of O-2A progenitors in the nerve, identified either by electron microscopy (Vaughn, 1969) or by quisqualate-induced cobalt uptake in whole nerves (Fulton et al., 1992).

In this paper, we have presented further evidence which corroborates our hypothesis that oligodendrocyte precursors originate in a precise region of the ventricular zone in the ventral half of the spinal cord. This evidence relies in part on our observation that mRNA encoding CNP is expressed, not only by differentiated oligodendrocytes but also, in smaller amounts, by proliferating oligodendrocyte progenitors in vitro. This is consistent with the results of another study showing that CNP mRNA and protein are expressed in the embryonic brain before the onset of myelination (Scherer et al., 1994) and suggests that the distribution of CNP mRNA during development might reflect the distribution of oligodendrocyte precursors. We visualized CNP mRNA in the developing rat spinal cord by in situ hybridization and found that the pattern of CNP transcripts in the E14 cord closely resembles the pattern of PDGF-αR transcripts at the same age. Both CNP and PDGF-αR are expressed in a small number of cells located at the ventricular surface, about 0.15 of the distance from the floor plate toward the dorsal tip of the central canal.

Outside of the CNS, the expression patterns of CNP and PDGF-αR are quite different. CNP appears to be restricted to the CNS and PNS at the ages we have examined, whereas PDGF-αR is expressed by a majority of cells in the embryo outside the CNS, including mesenchymal and neural crest-derived craniofacial tissues, but excluding the PNS. Within the embryonic CNS, there are also some differences between the patterns of CNP and PDGF-αR expression. CNP is expressed throughout the ventral half of the spinal cord at E12, presumably in neurons and their precursors, whereas PDGF-αR is not expressed in the ventral half of the cord until E14. PDGF-αR is weakly expressed in the dorsal ventricular zone of the spinal cord up to E14, presumably in a subset of neuroblasts, whereas CNP is not expressed in the dorsal half of the cord at these ages. Therefore, the only obvious overlap between CNP and PDGF-αR expression anywhere in the embryo between E12 and E14 is in the transient ventral column of presumptive oligodendrocyte precursors in the E14 spinal cord. Thus, the combined evidence now strongly suggests that this site represents the origin of the oligodendrocyte lineage in the embryonic spinal cord.

How are these latter few cells singled out from their neighbors that do not express CNP or PDGF-αR? It seems likely that, as in the establishment of body segments in the Drosophila embryo, a few early "pattern-forming" genes generate a relatively crude molecular prepattern which evolves into a more intricate pattern through cascades of interactions among genes and their products (Ingham, 1988). A similar progression in the spinal cord is suggested by the fact that CNP is first expressed throughout the ventral half of the spinal cord and that this broad expression domain subsequently collapses to a small focus in the ventral ventricular zone. Presumably, this reflects the expression of transcription factors that control the expression of many downsteam genes, including CNP. For example, members of the Pax family of transcription factors are differentially expressed along the dorsoventral axis of the developing spinal cord and might play a role in establishing pattern in the dorsoventral axis (Gruss and Walther, 1992).

PLP/DM-20 Is Expressed by Unidentified Precursor Cells in the Ventricular Zone Adjoining the Floorplate

DM-20, an alternatively spliced isoform of the myelin protein PLP is, like CNP, also expressed in the CNS before the appearance of myelinating oligodendrocytes (Timsit et al., 1992; Ikenaka et al., 1993). However,

unlike CNP, we did not detect PLP/DM-20 transcripts in cultured oligodendrocyte progenitor cells by Northern blot analysis. Consistent with this, we found that PLP/DM-20 transcripts did not colocalize with CNP transcripts in the embryonic CNS before the appearance of oligodendrocytes around E18. We found a focus of PLP/DM-20 expression in the ventral ventricular zone of the embryonic rat spinal cord, but at a different time and place than the focus of CNP/PDGF-αR expression. The focus of PLP/DM-20 expression is apparent at E12 and is gone by E14 when the PDGF-αR/CNP focus is most evident. We do not know what cells express PLP/DM-20 at E12. We found no evidence of PLP/DM-20 expression in the gray or white matter outside of the ventricular zone between E14 and E18, suggesting that PLP/DM-20 is transiently expressed by ventricular cells and is down-regulated when the cells mature into either neurons or glia. Motor neurons are still being generated in the ventral region of the spinal cord at E12, so one possibility is that the PLP/DM-20-expressing cells are motor neuron precursors. We think it is less likely that the PLP/DM-20-expressing cells are oligodendrocyte precursors because, as mentioned above, we did not detect significant quantities of PLP/DM-20 transcripts in purified O-2A progenitor cells in vitro. However, it remains a formal possibility that there may be two, or more, distinct lineages which give rise to oligodendrocytes in vivo.

Specialization within the Ventricular Germinal Zone

It has been suggested that the ventricular zone of the embryonic rat spinal cord is composed of a mosaic of specialized domains, each containing a different class of progenitor cell which generates a distinct subset of cell types (Wenger, 1950). Our data support this idea by demonstrating that there are distinct loci in the ventricular zone that express different sets of genes and presumably, therefore, represent cells that will follow different developmental paths. Two such loci are identified in the present paper. One of these, 0.15 of the distance from the ventral toward the dorsal tip of the central canal, is defined at E14 by expression of CNP and PDGF-αR. The other, just above the floor plate, is defined at E12 by expression of PLP/DM-20. It is probable that, given additional markers, further specializations in the ventricular zone would become apparent. It seems very likely that the site defined by expression of CNP and PDGF-αR is a source of oligodendrocyte precursors. Whether other classes of spinal cord glia or neurons are generated in equally restricted regions of the ventricular zone remains to be determined.

Experimental Procedures

O-2A Progenitor Cell Cultures

Our procedures for obtaining cultures of purified O-2A progenitor cells have been described in detail before (Collarini et al., 1992). Briefly, the method was as follows. Monolayer cultures of mixed glial cells were established by mechanical dissociation of neonatal rat cerebral cortices and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum until confluent. The cultures were removed from the culture dishes with trypsin and subjected to immunoselection once or twice with monoclonal antibody anti-RAN-2 (Bartlett et al., 1981) to remove astrocytes, then with monoclonal anti-galactocerebroside (Raff et al., 1978) to remove oligodendrocytes, and finally with monoclonal A2B5 (Eisenbarth et al., 1979) to positively select O-2A progenitors. The progenitors were removed from the final selection dish with trypsin and plated into 60 mm tissue culture dishes in modified Bottenstein–Sato defined medium (Richardson et al., 1988) containing 5 μg/ml insulin, 16 μg/ml putrescine, 10 ng/ml each of recombinant human PDGF-AA and recombinant human bFGF (Peprotech, NJ), and 0.5% fetal calf serum. After the cultures were semiconfluent, total cell RNA was prepared by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) from replicate cultures either before, or at various times after, growth factor withdrawal. Total cell RNA was analyzed on 1% agarose/2.2 M formaldehyde gels and subjected to Northern blotting and hybridization with ^{32}P-labeled DNA probes, generated by random priming. The CNP probe 1 (Figure 1A) was generated from a 370 bp EcoRI–HindIII fragment, and the CNP probe 2 was generated from an 850 bp PstI fragment of plasmid pCNP7 (Bernier et al., 1987), which contains a full-length rat cDNA. The PLP probe was generated from a 3 kb rat cDNA (Milner et al., 1985), kindly provided by Greg Lemke. The MBP and PK probes used were described previously (Collarini et al., 1992).

In Situ Hybridization

The in situ hybridization protocol was as described previously (Pringle and Richardson, 1993; Pringle et al., 1989). Embryos were aged according to the criteria described by Long and Burlingame (1938). The embryos were decapitated and fixed by immersion in 4% (w/v) paraformaldehyde in phosphate-buffered saline at room temperature for 2–3 hr or at 4°C overnight. The tissue was then placed in 0.5 M sucrose in phosphate-buffered saline at 4°C until it sank (usually overnight), frozen in OCT embedding medium (BDH), and stored at −70°C until needed. Cryosections (10 μm nominal thickness) were cut and collected on 3-aminopropyltriethoxysilane-coated glass slides for in situ hybridization. Single-stranded ^{35}S-labeled RNA probes ("sense" and "antisense") were generated by in vitro transcription with T7, T3, or SP6 bacteriophage RNA polymerase from DNA templates cloned into Bluescript (Stratagene) or pGEM (Promega) plasmid vectors. The PDGF-αR probe was the same as used previously (Pringle and Richardson, 1993). The CNP probe was transcribed from a subclone of plasmid pCNP7 (Bernier et al., 1987) in Bluescript, kindly provided by Art McMorris. The PLP/DM-20 probe was transcribed from a rat PLP cDNA (Milner et al., 1985), provided by Greg Lemke. All probes were reduced to ~150 bp fragments by partial hydrolysis before use. After development and micrography under dark-field illumination (in a Wild M3 dissecting microscope with dark-field/bright-field base), the sections were stained weakly with haemotoxylin and rephotographed under bright-field illumination. The dark-field and bright-field images were individually converted to digital format and imported into a Macintosh computer using a video camera and image grabber. Corresponding dark-field and bright-field images were then assigned false colors and superimposed using Adobe Photoshop software and photographed directly from the computer monitor.

Acknowledgments

This work was supported by grants from the Medical Research Council and the Multiple Sclerosis Society of Great Britain and Northern Ireland and by a Royal Society fellowship to W.-P. Y. from 1991 to 1992. We thank our colleagues at UCL for helpful comments and advice and John Kamholtz and Bernard Zalc for sharing their data prior to publication.

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Received December 29, 1993; revised March 24, 1994.

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